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Activating Intrinsic Carbohydrate-Active Enzymes of the Smut Fungus *Ustilago maydis* for the Degradation of Plant Cell Wall Components

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ABSTRACT

The microbial conversion of plant biomass to valuable products in a consolidated bioprocess could greatly increase the ecologic and economic impact of a biorefinery. Current strategies for hydrolyzing plant material mostly rely on the external application of carbohydrate-active enzymes (CAZymes). Alternatively, production organisms can be engineered to secrete CAZymes to reduce the reliance on externally added enzymes. Plant-pathogenic fungi have a vast repertoire of hydrolytic enzymes to sustain their lifestyle, but expression of the corresponding genes is usually highly regulated and restricted to the pathogenic phase. Here, we present a new strategy in using the biotrophic smut fungus *Ustilago maydis* for the degradation of plant cell wall components by activating its intrinsic enzyme potential during axenic growth. This fungal model organism is fully equipped with hydrolytic enzymes, and moreover, it naturally produces value-added substances, such as organic acids and biosurfactants. To achieve the deregulated expression of hydrolytic enzymes during the industrially relevant yeast-like growth in axenic culture, the native promoters of the respective genes were replaced by constitutively active synthetic promoters. This led to an enhanced conversion of xylan, cellobiose, and carboxymethyl cellulose to fermentable sugars. Moreover, a combination of strains with activated endoglucanase and β-glucanase increased the release of glucose from carboxymethyl cellulose and regenerated amorphous cellulose, suggesting that mixed cultivations could be a means for degrading more complex substrates in the future. In summary, this proof of principle demonstrates the potential applicability of activating the expression of native CAZymes from phytopathogens in a biocatalytic process.

IMPORTANCE

This study describes basic experiments that aim at the degradation of plant cell wall components by the smut fungus *Ustilago maydis*. As a plant pathogen, this fungus contains a set of lignocellulose-degrading enzymes that may be suited for biomass degradation. However, its hydrolytic enzymes are specifically expressed only during plant infection. Here, we provide the proof of principle that these intrinsic enzymes can be synthetically activated during the industrially relevant yeast-like growth. The fungus is known to naturally synthesize valuable compounds, such as itaconate or glycolipids. Therefore, it could be suited for use in a consolidated bioprocess in which more complex and natural substrates are simultaneously converted to fermentable sugars and to value-added compounds in the future.

One central aim of a sustainable bioeconomy is the switch from fossil- to bio-based production of platform chemicals and other valuable substances. To date, the most promising feedstock for biorefineries is lignocellulosic nonfood plant biomass (1). Lignocellulose is a complex composite mainly consisting of cellulose, hemicelluloses, pectin, and lignin (2). The selective conversion of lignocellulosic biomass comprises different steps: pretreatment to open up its recalcitrant structure, hydrolysis of hemicellulose and cellulose to release the fermentable sugars, and fermentation to convert the sugars into a valuable product (3, 4). Ideally, some or all of these steps would be performed in a single-pot consolidated bioprocess (5), provided that suitable microorganisms can be developed for such an approach. Strategies to engineer microorganisms for carbohydrate-active enzyme (CAZyme) (http://www.cazy.org/) (6) production include, e.g., optimizing and boosting the production of homologous enzymes, as well as the introduction of novel or complementary heterologous enzymes and pathways into a promising host (7–9).

Cellulose is the major component of lignocellulosic biomass and therefore constitutes the starting point for efficient biomass decomposition. At least three enzyme types acting synergistically are necessary for its degradation: endoglucanase, exoglucanase or cellobiohydrolase (CBH), and β-glucosidases. Within the heterogeneous group of hemicellulose polysaccharides, xylan represents the major fraction. It can be substituted with arabinose, glucuronic acid, or other hexose sugars. Due to this complexity, various enzymes are needed for complete degradation here as well. These include endoxylanase, β-xylosidase, arabinofuranosidase, feruloyl esterase, and α-glucuronidase (10). Filamentous fungi, like *Trichoderma reesii* (*Hypocrea jecorina*), *Aspergillus niger*, and *Myceliophthora thermo-*

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phila, are among the best known fungal CAZyme producers (10, 11). Due to the presence of potent polysaccharide monooxygenases, which aid in the cellulose degradation process, *Neurospora crassa* has recently been added to the list (10). Bacteria, such as *Clostridia*, use an alternative successful strategy for cellulose degradation by producing cellulosomes (12). These have been adopted for expression in eukaryotes and also have been artificially modified to degrade lignocellulosic biomass (13).

Different microorganisms are currently established for consolidated bioprocessing using renewable feedstock, including bacteria, yeasts, and filamentous fungi, with some processes running in pilot-scale facilities (3). For bioethanol production, the conversion of lignocellulosic biomass by eukaryotic microorganisms is already being developed industrially (4, 14–16). However, there is a growing demand for alternative chemicals for the production of polymers, high-performance biofuels, and other valuable substances for industrial applications. Organic acids, for example, are considered bio-based substitutes for traditional petroleum-derived products (17, 18). Among these, itaconic acid is a promising and versatile building block, i.e., for the production of polymers, adhesives, and coatings, and in the future possibly also for the generation of biofuels (19–21). Commercial itaconic acid production is currently achieved biotechnologically with engineered strains of *Aspergillus terreus*. Although this filamentous fungus is remarkably efficient in terms of its itaconic acid yield and titer (22, 23), it is worthwhile to work on alternative unicellular production organisms since they potentially enable a more efficient and controllable high-density process (24, 25).

Members of the *Ustilaginaceae* smut fungi are promising natural producers of organic acids (21, 26, 27). Within this group of biotrophic plant pathogens, *Ustilago maydis* is of increasing interest, not least due to its long history of intensive investigation as a paradigm of plant-pathogen interaction (28). Accordingly, *U. maydis* is best known for its pathogenicity toward maize (*Zea mays*), where it causes corn smut (29, 30). Interestingly, infected corn is a delicacy in Mexico and has been consumed by humans for hundreds of years, indicating its innocuousness (26).

Certain *U. maydis* strains, such as the wild-type isolate MB215, can produce high concentrations of itaconate as well as other compounds, such as malate, succinate, hydroxyparaconate, and erythritol (21, 31), some of which are considered top value-added platform chemicals (17). Remarkably, itaconic acid synthesis in *U. maydis* wild-type isolates already yields about twice as much as *A. terreus* strains did in early development, underpinning the potential of this organism and the resulting need for further research into this direction (24). Further interesting secondary metabolites found in *U. maydis* are the glycolipid biosurfactants mannosylerythritol and cellobiose lipids, which could be applied, e.g., in the food industry, medicine, and pharmacology, or even in agriculture and remediation (32–37).

U. maydis exhibits filamentous growth during plant infection and colonization. However, it can also grow saprotrophically as unicellular haploid yeast and proliferates in simple medium in axenic culture with various hexoses and pentoses as a carbon source (25, 38). In the yeast form, the fungus is nonpathogenic and amenable to genetic manipulation. Due to the broad knowledge on its infection strategy, it is simple to generate safe and nonpathogenic strains lacking, for example, the mating type genes (30). The genome is sequenced and manually annotated (29). Numerous molecular tools have been developed over the last few years, including resistance markers, protein tags (39), and efficient strain generation by Golden Gate cloning (40). In addition, in bioreactors, the fungus confers a remarkably high resistance to shearing forces and to impurities in the fermentation broth, like those observed after biomass pretreatment (26, 41). The large-scale cultivation in bioreactors is well established, for instance, for the closely related fungus *Pseudozyma tsukubaensis* (42).

As a plant pathogen, *U. maydis* possesses a limited but complete set of hydrolases that in theory enable the utilization of complex substrates, like cellulose, xylan, or pectin, for growth (21, 38, 43, 44). However, transcription studies revealed that gene expression for most of these CAZymes is restricted to the phase of infection, when the fungus invades the plant and proliferates, while they are often dormant during the industrially relevant yeast-like growth (45). Therefore, the aim of this study is to provide a proof of principle for overcoming this limitation by improving the saccharification of plant cell wall components by the yeast form using deregulation of potent intrinsic enzymes. Ultimately, the resulting sugars should directly be fermented to organic acids, such as itaconic acid. To prove the feasibility of this approach, the strategy was applied to different (hemi)cellulolytic enzymes, i.e., endoxylanase, β-glucanase, and endocellulase, using simple cell wall-derived substrates.

MATERIALS AND METHODS

Plasmids and plasmid constructions. For the generation of plasmid vectors, standard molecular cloning techniques were used (46). Plasmids harboring gene activation constructs were generated by Golden Gate cloning (40) (see Table S1 in the supplemental material). To this end, flanking regions of about 1 kb directly up- and downstream of the promoter region of the target gene were generated by PCR or obtained as gBlocks (IDT, Belgium). Genomic DNA of strain UM521 (DSM 14603) was used as a template. The PCR products were purified by standard procedures (e.g., SureClean, Bioline; JetSorb, Genomed). To generate destination vectors containing the gene activation constructs, BsaI-mediated Golden Gate reaction mixtures containing the two respective PCR products (flanks), a storage vector, and the destination vector (pDestI/pUMa1467) were made as described elsewhere (40). Plasmids pStor1_2-4 h (pUMa1507 [40]) and pStorI_2-5n (pUMa2326, see below) served as storage vectors harboring a hygromycin resistance (HygR)/P_{otef} and a nourseothricin resistance (NatR)/P_{oma} resistance cassette module, respectively, for constitutive expression of the target gene. The exact components of all Golden Gate-derived vectors are listed in Table S1. The oligonucleotides used for flank generation are described in Table S1, and the corresponding sequences are displayed in Table 1.

For promoter replacements with the constitutive active promoter P_{oma}, a novel BsaI-compatible storage vector was generated, designated pStorI_2-5n (pUMa2326). For that purpose, the 2.5-kb backbone of pUMa1778 (pStorI_2-3 h [40]) was isolated by SfiI restriction, and the 1.4-kb NatR cassette of pUMa326 (pMF3-4n [39]) was generated by restriction with XbaI/SfiI. Both parts were combined with a PCR fragment containing P_{oma} in a three-fragment ligation. The 1.3-kb PCR product containing P_{oma} was amplified from pUMa2113 (pRabX1PomaGus-SHH-Cts 1 ubi1 3′ untranslated region [UTR] [47]) using primers oRL1855 and oRL1856 and digested with XbaI and SfiI to be compatible for ligation.

Strains and promoter replacements. The *Escherichia coli* K-12 derivate TOP10 (Invitrogen/Life Technologies, Darmstadt, Germany) was used for cloning purposes. The cells were grown at 37°C with 200-rpm shaking in glass tubes.

The *U. maydis* strains used in this study are displayed in Table 2 and Table S2 in the supplemental material. Cells were incubated at 28°C with 200-rpm shaking. Cultures were grown in complete medium (48) supple-

TABLE 1 Sequences of DNA oligonucleotides used in this study

Designation	Nucleotide sequence (5′–3′)
oDD19	CATGTACGCCGGTATCTCG
oDD20	CTCGGGAGGAGCAACAATC
oDD70	GGTCTCCGGCCATGATGGCCACCGTCAAGTCGCTGC
oDD71	GGTCTCGCTGCAATATTTTGTTCGTAAGCGAGGAAGTGCTTTGC
oDD72	GGTCTCGCCTGCAATATTGTGCTCACGCTTACTTCATTAGC
oDD73	GGTCTCCAGGCCGGTTGACAGCATAAATACATACTGG
oDD76	GGTCTCGCCTGCAATATTCATTTCAGAGTCGAACAGGG
oDD77	GGTCTCCAGGCCGATGAGCTACCAGCTACCAGTCTCTGC
oDD137	GGTCTCGCCTGCAATATTGAAGACGGCGTGCAAGTAAGC
oDD138	GGTCTCCAGGCCTGCTGGATCGTCGTTGCACTCTGG
oDD139	GGTCTCCGGCCATGGTGTTTTCTTCTCACAAGTCAACC
oDD140	GGTCTCGCTGCAATATTCACGATGGCTTTTGGGATTACACG
oDD178	GGTCTCGCCTGCAATATTTCAGCATTCCAGCAAAAGTGTCTAACG
oDD179	GGTCTCCAGGCCGTCACATGAAACGCATGTTGTAATGC
oDD180	GGTCTCCGGCCATGGCCTTCAAGCTCAACATCG
oDD181	GGTCTCGCTGCAATATTGCTGCCGCCCGAAGGAGGTGC
oDD921	CTCGGAACTGCTCAACAATCG
oDD922	CATCACCAGGCATGGTCATATC
oDD923	CACGCTGTTCTACCAAGTCATC
oDD924	GAAATATCGCTGCCCTTCCAC
oDD927	CCTTCGGGAGGAAATGATCAG
oDD928	CGTCGCTACCAGAAGTAGGAG
oDD929	CAAACCCTCCCGACTTTGTTG
oDD930	CGGATTGTTGAGCCATCCATAG
oRL1855	CACCACTCTAGAGGCCTACTTCTCGAGCAGGGGG
oRL1856	GTGGGCCACTCAGGCCGGGGATCCTGATAGAGTAAGG
oRL1901	GGTCTCGCCTGCAATATTGGTTAGGATCTTCACAGTGC
oRL1902	GGTCTCCAGGCCTTGCATGCTGTTTCTCGGTTGG
oRL1903	GGTCTCCGGCCATGAAGTTTGCCACTGTCC
oRL1904	GGTCTCGCTGCAATATTCTTGCACACACTTCACC

mented with 1% (wt/vol) glucose (CM-Glc), or as described below for the growth experiments, secretome preparation, and enzyme assays.

U. maydis MB215 promoter replacement mutants were generated by homologous recombination, according to established protocols (39, 40, 49). The mutants were obtained by transformation of the progenitor strain MB215 (DSM 17144; wild-type isolate described in reference 50) with linear constructs consisting of flanking regions and an antibiotic resistance cassette (Fig. 1). To this end, the relevant replacement constructs were excised from the corresponding vectors using the blunt-end enzyme SspI (see Table S1 in the supplemental material) (40). The genes of interest were replaced by either a hygromycin or a nourseothricin resistance cassette (HygR or NatR, respectively; Table 2). Promoter replacement by homologous recombination was verified by Southern blotting (51).

Quantitative real-time PCR. Total RNA was extracted from the respective strains grown in screening medium (21) supplemented with 20 g liter^{-1} glucose for 12 h using the Tri Reagent (Roth, Karlsruhe, Germany), according to the manufacturer's instructions. Ten micrograms of total RNA was treated with 2 U of DNase I (New England BioLabs, Frankfurt am Main, Germany) for 10 min at 37°C to remove residual genomic DNA and subsequently purified by phenol-chloroform extraction and sodium acetate precipitation. cDNA synthesis was performed with the SuperScript III first-strand synthesis SuperMix (Invitrogen) using oligo(dT)$_{20}$

TABLE 2 *U. maydis* MB215 strains used in this study[a]

Strain designation (UMa strain collection no.)	Relevant genotype and resistance information	Plasmid transformed, resistance (pUMa no.)	Manipulated locus (annotation of the encoded enzyme/predicted GH family)[b]	Reference or source
MB215 (UMa1160)	*a2b13*, no resistance (wild-type isolate; progenitor strain)			DSM 17144, 50
MB215Δxyn11A	Δ*um06350*, HygR	pMF1_Δumag_06350, HygR	*umag_06350* (endoxylanase Xyn11A/GH11)	54
MB215P$_{oma}$bgl1 (UMa1535)	ΔP_{00446}::*umag_00446*::P_{oma}::*umag_00446*, NatR	pDest_Poma::umag_00446, NatR (pUMa2428)	*umag_00446* (β-glucanase Bgl1/GH3)	This study
MB215P$_{oma}$egl1 (UMa1636)	ΔP_{06332}::*umag_06332*::P_{oma}::*umag_06332*, NatR	pDest_Poma::umag_06332, NatR (pUMa2541)	*umag_06332* (endoglucanase Egl1/GH45)	This study
MB215P$_{oma}$xyn11A (UMa1474)	ΔP_{06350}::*umag_06350*::P_{oma}::*um06350*, NatR	pDest_Poma::umag_06350, NatR (pUMa2348)	*umag_06350* (endoxylanase Xyn11A/GH11)	This study

[a] Activated strains that did not display detectable enzymatic activities are listed in Table S2 in the supplemental material.
[b] Per the Nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide).

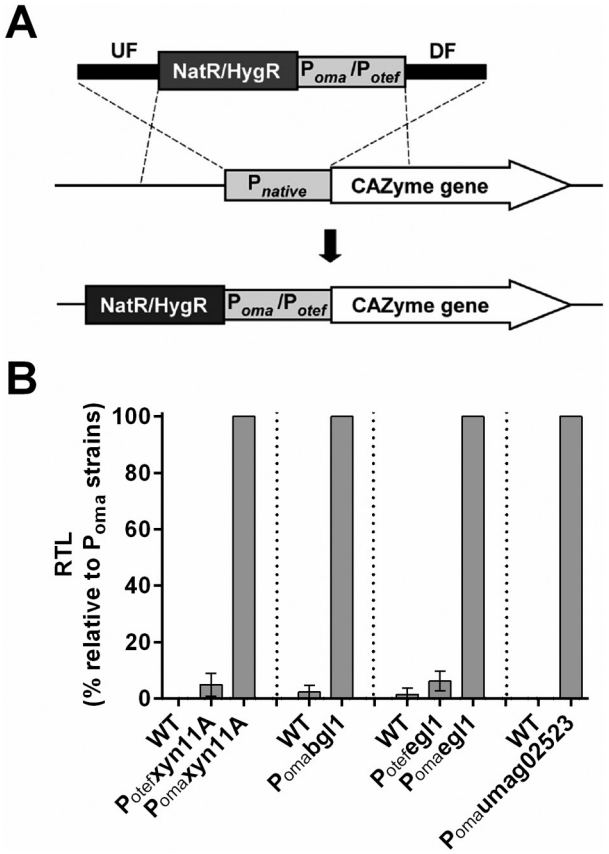

FIG 1 Promoter replacements. (A) Schematic representation of *in-locus* promoter replacements using homologous recombination. Flanking regions with homology to the 5′ region of the native promoter (UF, upstream flank) and the 5′ region of the target gene (DF, downstream flank) are depicted as black boxes. The respective modules are stably integrated into the genome. The native promoter of a CAZyme-encoding gene is replaced by either the synthetic promoter P_{oma} using an upstream nourseothricin resistance cassette (NatR) or by the synthetic promoter P_{otef} using an upstream hygromycin resistance cassette (HygR) as a selective marker. (B) Transcriptional profiling of overexpression mutants with activated CAZyme genes using qRT-PCR. Relative transcript levels (RTLs) were calculated using the ΔC_T method, and the RTLs of the P_{oma} strains were set to 100%. Error bars represent standard deviations. The experiment has been performed in three biological replicates.

primers and 0.5 μg of DNase I-treated total RNA as a template. Ten microliters of the 1:10 dilution of cDNA was used for one quantitative real-time PCR (qRT-PCR) conducted with the my-Budget 5× EvaGreen quantitative PCR (qPCR)-mix II (ROX) (Bio-Budget) in an Mx3000 cycler (Stratagene). For each strain, three biological replicates were assayed by qPCR in three technical replicates each, using gene-specific primers (*umag_06350*, oDD929xoDD930; *umag_00446*, oDD921xoDD922; *umag_06332*, oDD927xoDD928; and *umag_02523*, oDD923xoDD924). The specificity of the RT-PCR products was documented beforehand by standard gel electrophoresis and resulted in single products. The housekeeping gene *actin* was used for normalization (*umag_11232*, oDD19xoDD20). cDNA synthesis reaction mixtures lacking reverse transcriptase were included as a template to exclude contaminations with genomic DNA (gDNA). mRNA levels were calculated as means of relative transcript levels (RTLs) to the *actin* transcript using the ΔC_T method (C_T, threshold cycle). The oligonucleotide efficiency was taken into account using previously published methods (52). Due to large variations between biological replicates but identical trends of strong upregulation in P_{oma} strains, these values were set to 100% RTL.

Growth experiments. Growth experiments of *U. maydis* strains (Table 2) were performed in the Duetz-System (24-well plates) with a filling volume of 1.5 ml (shaking diameter, 50 mm; agitation speed, 300 rpm; temperature, 30°C; relative air humidity, 80%) (53). The medium for growth experiments and organic acid production contained 0.8 g liter^{-1} NH_4Cl, 0.2 g liter^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g liter^{-1} $FeSO_4 \cdot 7H_2O$, 0.5 g liter^{-1} KH_2PO_4, 1 ml liter^{-1} vitamin solution, and 10 ml liter^{-1} trace element solution. The compositions of the vitamin and trace element solutions have been described previously (21). As a carbon source, 50 g liter^{-1} glucose, cellobiose (Sigma-Aldrich, Schnelldorf, Germany), or xylan from beech wood (Carl Roth, Karlsruhe, Germany) was used. All cultures were buffered with 19.5 g liter^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES), except for the organic acid production cultures on cellobiose, which were buffered with 33 g liter^{-1} $CaCO_3$. The pH of the MES stock solution was adjusted to 6.5 with NaOH. Growth experiments with Xyn11A overexpression mutants were performed on minimal medium, as described previously (54), with 20 g liter^{-1} xylan from beech or birch wood (Carl Roth). Cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.5.

Secretome preparation and enzyme assays. For the isolation of culture supernatants containing secreted proteins (secretome), strains were grown in the medium described above, with 1.6 g liter^{-1} NH_4Cl and 20 g liter^{-1} glucose to avoid the presence of residual reducing groups. After cultivation for 24 h, 1 ml of culture broth was filtered with Rotilabo (Carl Roth, Karlsruhe, Germany) syringe filters (cellulose acetate [CA], 0.20 μm; diameter, 15 mm). The freshly isolated supernatants were used for the enzymatic assays.

The substrates used in this study were 1% (wt/vol) cellobiose (Sigma-Aldrich, Schnelldorf, Germany), 1% (wt/vol) xylan from beech or birch wood (Carl Roth, Germany), 1% (wt/vol) Avicel PH-101 (microcrystalline cellulose; Sigma-Aldrich), 1% (wt/vol) carboxymethyl cellulose (CMC) sodium salt purum (Fluka; Sigma-Aldrich), and approximately 0.7% (wt/vol) regenerated amorphous cellulose (RAC) dissolved in 50 mM sodium acetate buffer (pH 5). RAC was prepared according to Zhang et al. (2006) from Sigmacell cellulose S3504 type 20, 20 μm (Sigma-Aldrich) (55). Secretome samples of 200 μl were incubated with 800 μl of substrate solution at 37°C and 600 rpm in an HLC cooling Thermo shaker MKR 13 (Ditabis AG, Pforzheim, Germany). At different time points, 60-μl samples were taken for analysis. The concentrations of the reducing groups were determined immediately by a 3,5-dinitrosalicylic acid (DNS) assay (reference 56 and see below). Hydrolysis products were determined by high-performance liquid chromatography (HPLC) analysis, after stopping the enzymatic reaction with 50 μl of 20% (vol/vol) H_2SO_4. One enzyme unit was defined as a 1-μmol concentration of reducing groups (with glucose or xylose as a standard) released per milligram of enzyme per minute under the applied assay conditions.

Analytic methods. Cell densities were determined with an absorption of 600 nm using a 1201 spectrophotometer (4-ml cuvettes, 1-cm path length; Unico, Dayton, OH, USA). Alternatively, a Synergy Mx multimode plate reader (BioTek, Winooski, VT, USA) was used with 24-well plates (Costar cell culture plates; Sigma-Aldrich, Schnelldorf, Germany) with a 400-μl filling volume. The plate reader values correlate with the cuvette photometer values with a correlation factor of 20.4. Before measuring cell densities, calcium carbonate was dissolved by adding 10 μl of HCl per 100 μl of sample.

The concentration of reducing groups was determined by a 3,5-dinitrosalicylic acid (DNS) assay (56). Sixty microliters of fresh working solution (5 g of DNS and 202 g of sodium potassium tartrate tetrahydrate dissolved in 0.5 liter of 0.4 N sodium hydroxide) was added to a 60-μl sample containing at most a 20 mM concentration of reducing groups, and the mixture was boiled at 94°C for 10 min. After cooling on ice, reducing sugar concentrations were determined by measuring the absorbance at 540 nm with a Synergy Mx multimode plate reader (BioTek, Winooski, VT, USA). Standard curves were prepared using appropriate amounts of xylose or glucose in the range of 0 to 20 mM.

Glucose, cellobiose, itaconate, malate, succinate, xylose, xylotriose, and xylobiose concentrations in culture supernatants were determined by HPLC (Beckmann Coulter GmbH, Germany), as described earlier (21). Xylan degradation products were analyzed by thin-layer chromatography (TLC), as previously described (21).

The protein concentration in the supernatant was determined by using the 96-well plate assay protocol for Bradford reagent (B6916; Sigma-Aldrich, Germany) and bovine serum albumin (BSA) standards ranging from 0 to 1.4 g liter^{-1}, according to the manufacturer's manual.

RESULTS

Exploiting constitutive synthetic promoters for deregulation of CAZyme expression. The majority of the lignocellulose-degrading enzymes in *U. maydis* (38, 43, 44) are specifically expressed and secreted during biotrophic plant-dependent growth (29, 45). To activate the expression of individual CAZyme genes during the biotechnologically relevant yeast-like growth in axenic culture, the native promoters of candidate genes were replaced by two different synthetic promoters, termed P_{otef} (57) and P_{oma} (58, 59), using homologous recombination (57, 58) (Fig. 1A). These two constitutive promoters were previously described to mediate high (P_{otef}) and very high (P_{oma}) gene expression during axenic growth (47). A Golden Gate cloning strategy was applied in which flanking regions of about 1 kb in length were combined with a resistance cassette module containing the respective artificial promoter (40). Taking into account the arrangement of neighboring genes at the genomic locus, the region upstream of the start codon of the gene of interest was replaced by a construct harboring the respective synthetic promoter and a hygromycin or nourseothricin resistance cassette (HygR or NatR, respectively; Fig. 1A). In this way, promoter exchange vectors for four genes were constructed (see Table S1 in the supplemental material). However, not all constructs could be successfully integrated into the respective genomic loci of the *U. maydis* isolate MB215. Overexpression mutants in which at least one of the two promoters was inserted at the respective loci were obtained for the putative CAZyme genes *umag_06350* (encoding the endo-1,4-β-xylanase Xyn11a, glycoside hydrolase [GH] family 11), *umag_00446* (encoding a predicted β-glucanase, GH family 3), *umag_06332* (encoding the endoglucanase Egl1, GH family 45), and *umag_02523* (encoding a predicted endoglucanase, GH family 45) (GenBank accession no. NC_026478) (Table 2; see also Table S2 in the supplemental material) (54, 60).

To first test if the promoter replacements do in fact lead to the expected enhanced transcript levels in the overexpression mutants compared to the wild-type strain, quantitative real-time PCR experiments were conducted for each targeted gene (Fig. 1B). In line with the published observation that CAZyme genes are mostly silent during yeast-like growth, the abundance of the respective transcripts in the wild-type strain was very low for each tested gene. We detected large variations in the induced expression levels between the biological replicates, which can likely be attributed to this very low expression of the wild type (WT) used as a baseline. For genes that were controlled by the P_{otef} promoter, the transcriptional induction was unexpectedly low compared to the induction by P_{oma} (Fig. 1B). Importantly, promoter replacements with P_{oma} led to a very strong transcriptional induction in all cases, with fold changes in the range of 10^2 to 10^4 magnitudes relative to the level of transcript in the wild type. Thus, we succeeded in generating six strains that overexpress CAZyme genes from the homologous loci, with four of them showing strongly elevated transcription levels associated with the P_{oma} promoter (Table 2 and Fig. 1B; see also Table S2 in the supplemental material).

Artificial activation of CAZymes during yeast-like growth. To investigate if the promoter replacements also influenced CAZyme activities in the yeast phase, the six strains were analyzed with respect to the degradation of suitable lignocellulose-derived substrates. Therefore, overexpression mutants and the wild type were cultivated in minimal medium with glucose. After 24 h of incubation, cell-free culture supernatants were harvested, mixed with the relevant substrate, and analyzed for the accumulation of the respective monosaccharides over time (Fig. 2). The formation of reducing groups from xylan and CMC was determined by DNS assays and TLC. Accumulation of glucose from cellobiose and Avicel was determined by a HPLC-reactive index (RI) detector.

First, we concentrated on hemicellulose degradation by the endoxylanase Xyn11A. This enzyme had been characterized in an earlier study and identified as a key player in xylan degradation (54). Of note, Xyn11A is present in yeast cell cultures containing xylan as a carbon source, and a respective deletion mutant MB215Δxyn11A was available (54). The hemicellulose-depolymerizing activity of the MB215 wild-type, MB215Δxyn11A, and MB215P_{oma}xyn11A culture supernatants was analyzed using beech wood xylan. Xylanase activity in MB215Δxyn11A increased only very slightly during the assay, suggesting that Xyn11A is the main active xylanase under these conditions. The overexpressing mutant MB215P_{oma}xyn11A in fact had a 4- and 14-fold increased xylanase activity compared to MB215 and the *xyn11A* deletion mutant, respectively (Fig. 2A; MB215, 2.30 ± 0.14 U; MB215Δxyn11A, 0.67 ± 0.28 U; MB215P_{oma}xyn11A, 10.12 ± 1.89 U). In contrast, the mutant expressing *xyn11A* under the control of P_{otef} did not reveal any increase in xylanase activity (MB215P_{otef}xyn11A, 2.02 ± 0.84 U). This observation is likely a consequence of the relatively weak transcriptional induction that was achieved using this promoter (Fig. 1B). As a control, to ensure that the low xylanase activity is not caused by a rendered protease activity, all secretomes were incubated either with or without protease inhibitor cocktail for 1 h prior to the assay, yielding similar activities (data not shown). These results demonstrate that the promoter replacement in the overexpression mutant MB215P_{oma}xyn11A led to a strong increase in active secreted Xyn11A, suggesting that promoter replacements are a good means to activate CAZymes in the yeast phase.

Cellulose is the major component of most lignocellulosic biomass (61). Therefore, in the next step, we focused on cellulose-degrading enzymes. Importantly, unlike on xylan, wild-type *U. maydis* MB215 is unable to grow on cellulose or its oligosaccharides in its yeast form.

First, the expression and secretion of the predicted β-glucosidase termed Bgl1 that had been activated using the P_{oma} promoter (Fig. 1B) were analyzed. Glucosidase activity was determined by incubating culture supernatants of MB215 and the activated strain MB215P_{oma}bgl1 with 1% (wt/vol) cellobiose and determining the concentration of the substrate and its hydrolysis product (glucose) over time (Fig. 2B). Culture supernatants of MB215P_{oma}bgl1 indeed accumulated glucose and displayed a glucosidase activity of 1.04 ± 0.25 U, while the progenitor strain MB215 (WT) did not show any detectable activity under these conditions. Thus, in contrast to the wild type, the overexpression mutant MB215P_{oma}bgl1 is able to hydrolyze cellobiose.

Similarly, the transcription of two endoglucanase genes encod-

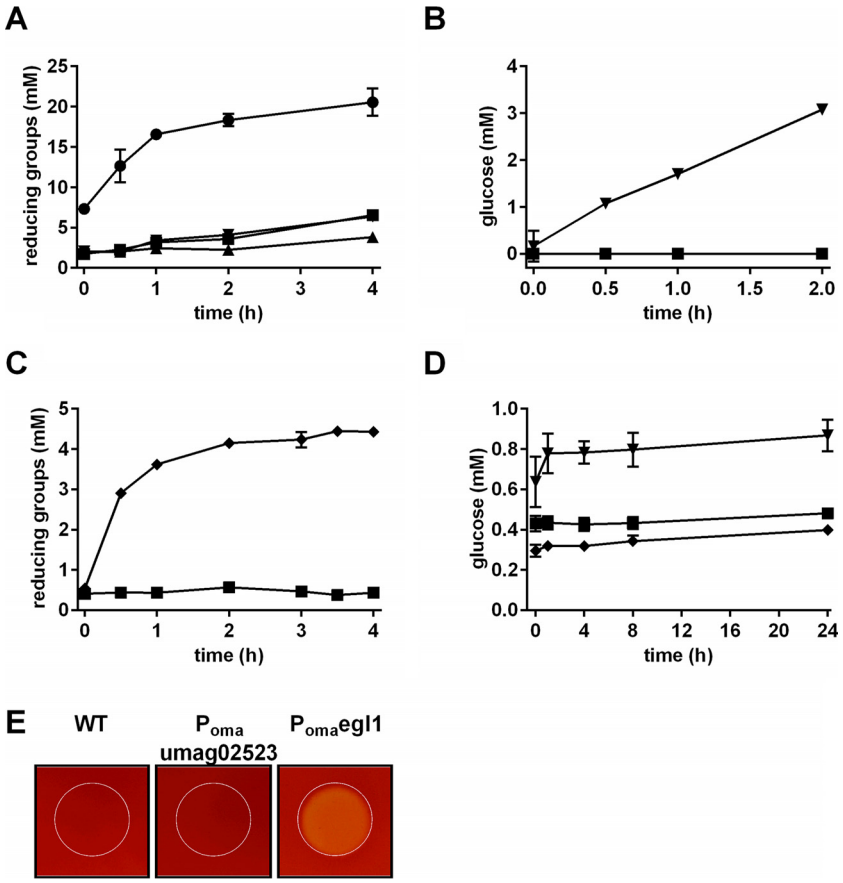

FIG 2 Enzyme activities of activated strains on different substrates. Analysis of cell-free supernatants from cultures grown with glucose as carbon source. Values are the arithmetic mean of three biological determinations. Error bars indicate deviation from the mean ($n = 3$). (A) Xylan hydrolysis by MB215 derivatives with activated *xyn11A* expression. Concentration of reducing groups released from beech wood xylan by strain MB215 (■), the *xyn11A* deletion mutant (▲), and the MB215P$_{otef}$xyn11A (▼) and MB215P$_{oma}$xyn11A overexpression mutants (●). (B) Degradation of cellobiose after constitutive activation of a β-glucosidase. Concentration of glucose during incubation of culture supernatants of the MB215 wild type (■) and a MB215P$_{oma}$bgl1 overexpression strain (▼) with 1% (wt/vol) cellobiose. (C) Degradation of CMC after constitutive activation of endocellulase Egl1. Concentration of reducing groups released from secreted enzymes of MB215 wild type (■), and the corresponding MB215P$_{oma}$egl1 overexpression mutant (◆), during incubation in 1% (wt/vol) CMC solution. (D) Degradation of Avicel after constitutive activation of cellulolytic enzymes. Concentration of glucose during incubation of culture supernatants of the MB215 wild type (■), MB215P$_{oma}$bgl1 (▼), and MB215P$_{oma}$egl1 overexpression strain (◆) with 1% (wt/vol) Avicel. (E) CMC degradation was confirmed using a Congo red assay on CMC-containing plates.

ing Umag_02523 and Egl1 was artificially activated. While the functional annotation of Umag_02523 is solely based on bioinformatic predictions (44), the endoglucanase activity of Egl1 has been demonstrated before (60). Supernatants of the corresponding MB215 derivatives MB215P$_{oma}$umag_02523 and MB215P$_{oma}$egl1 were assayed for enzymatic activity using CMC, a well-accepted substrate to assay endoglucanase activity individually (62). MB215P$_{oma}$umag_02523 supernatants did not reveal any endocellulase activity under these assay conditions (data not shown), although the corresponding qRT-PCR results of this strain showed a transcriptional induction compared to very low transcript levels in the wild type (Fig. 1B; fold changes in the magnitude of 10^3 to 10^5 compared to the wild type). In contrast, for the overexpression mutant MB215P$_{oma}$egl1, the enzyme assay using culture supernatants showed an endocellulase activity of 0.087 ± 0.007 U, which is 15-fold higher than the activity in the progenitor MB215 (0.006 ± 0.009 U) (Fig. 2C). Consistently, the activity could also be confirmed in a Congo red plate assay (60, 63). In contrast, a strain in which P$_{otef}$ was used to express *egl1* revealed only wild-type activity (0.004 ± 0.002 U; not shown). This finding is in line with the lower activity of this promoter (47) and the observations made for Xyn11A.

As an alternative and more complex substrate, the microcrystalline cellulose Avicel was included in the analysis. While strain MB215P$_{oma}$egl1 was unable to generate glucose, unexpectedly, we observed that MB215P$_{oma}$bgl1 was able to produce small amounts of glucose from this substrate (Fig. 2D). Hence, in total, enzyme activities could be verified for two of three activated cellulolytic CAZymes, the β-glucanase Bgl1 and the endoglucanase Egl1.

Synergistic effects of mixed supernatants. After the successful individual activation of the β-glucosidase Bgl1 as well as the endoglucanase Egl1, we aimed to test the potential synergistic effects of mixed secretomes of the respective strains. To achieve this, the enzyme activities of the 1:1 mixed-culture supernatants from MB215P$_{oma}$bgl1 and MB215P$_{oma}$egl1 were assayed on CMC (Fig. 3A and B). As an additional and more natural substrate, regenerated amorphous cellulose (RAC) was also included in the analysis (Fig. 3C and D). As controls, the secretomes of the

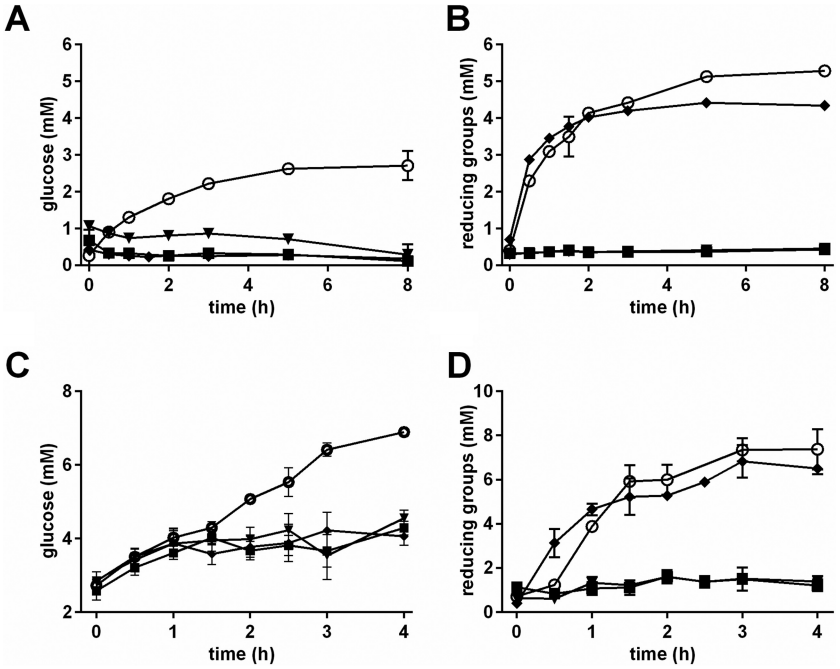

FIG 3 Degradation of CMC (A and B) and RAC (C and D) after constitutive activation of β-glucosidase Bgl1 and endocellulase Egl1. Concentration of glucose (A and C) and reducing groups (B and D) during incubation of culture supernatants of the MB215 wild type (■), MB215P$_{oma}$bgl1 (▼), and MB215P$_{oma}$egl1 overexpression strain (◆) and their 1:1 mixture (○) with 1% (wt/vol) CMC and 0.7% (wt/vol) RAC, respectively.

wild type as well as the single activated strains were used. Indeed, in contrast to all controls, the mixed samples of MB215P$_{oma}$bgl1 and MB215P$_{oma}$egl1 produced glucose from CMC and RAC (Fig. 3A and C). Interestingly, in both cases, the major amount of reducing sugars produced can be attributed to the activity of MB215P$_{oma}$egl1 alone (Fig. 3B and D). Nevertheless, the accumulation of glucose in the strain mixtures suggests that the two enzymes exhibit synergistic actions. Hence, a suitable combination of CAZyme-activated strains is a promising strategy toward lignocellulose degradation.

In contrast, the combined secretomes of MB215P$_{oma}$bgl1 and MB215P$_{oma}$egl1 only released minimally elevated amounts of glucose from Avicel compared to the controls under these assay conditions (not shown). The results suggest that more enzymes need to be activated to allow the sufficient degradation of more complex substrates.

Growth on (hemi)cellulosic substrates and production of valuable products. The fact that elevated enzyme activities were detected in different activated strains does not necessarily mean that the overexpression mutants can also grow on the respective substrates. Furthermore, the production of valuable products, such as organic acids, requires specific conditions (i.e., nitrogen limitation) that may not be compatible with CAZyme activation conditions. Hence, in the next step, the activated strains were tested for growth on the respective enzyme substrates as a single carbon source and the simultaneous production of the organic acids itaconate and malate.

First, the endoxylanase-producing strain MB215P$_{oma}$xyn11A was investigated with regard to one-pot hemicellulose depolymerization, growth, and acid production. Indeed, beech wood xylan degradation of MB215P$_{oma}$xyn11A starts earlier and displays a higher degradation rate than in the corresponding progenitor strain MB215, as suggested by the faster increase of reducing groups (Fig. 4A). This result was further confirmed by the visualization of xylan hydrolysis products by TLC over time, revealing an enhanced production of smaller xylan oligomers (Fig. 4B). The earlier degradation was reflected by a shorter lag phase of the overexpression strain (approximately 6 h) in comparison to MB215 wild type (approximately 12 h) (Fig. 4C), indicating that the constitutive gene expression leads to an earlier accumulation of the responsible xylanase in the culture supernatant. The growth rate of the strain was not affected (overexpression mutant, 0.011 ± 0.003 h^{-1}; MB215, 0.012 ± 0.002 h^{-1}). After 4 days of cultivation, 0.08 ± 0.01 g $liter^{-1}$ itaconate, 0.96 ± 0.02 g $liter^{-1}$ malate, and various glycolipids (not shown) were produced by the *xyn11A* overexpression mutant. Although this is in a range similar to that of the wild-type cultivations, it shows that the activated strain is faster in converting the released sugars from beech wood xylan to valuable products.

We then analyzed growth of MB215P$_{oma}$bgl1 on cellobiose. Indeed, the effect of constitutive *bgl1* overexpression was even more pronounced and not only mediated cellobiose degradation in enzyme assays but also enabled *U. maydis* to grow on cellobiose as a sole source of carbon (Fig. 5A). During the first 48 h of cultivation, cellobiose was hydrolyzed to glucose (Fig. 5B) with concurrent growth. When using medium suitable for the production of organic acids, the cellobiose was eventually converted to 10.3 ± 0.86 g $liter^{-1}$ malate (0.2 ± 0.02 g_{malate} $g_{cellobiose}^{-1}$) and 5.2 ± 0.22 g $liter^{-1}$ itaconate (0.1 ± 0.01 $g_{itaconate}$ $g_{cellobiose}^{-1}$) (Fig. 5C). Other products, such as mannosylerythritol and cellobiose lipids, were also observed (not shown). Hence, our strategy indeed enabled the production of organic acids from cellobiose in the yeast phase of *U. maydis*.

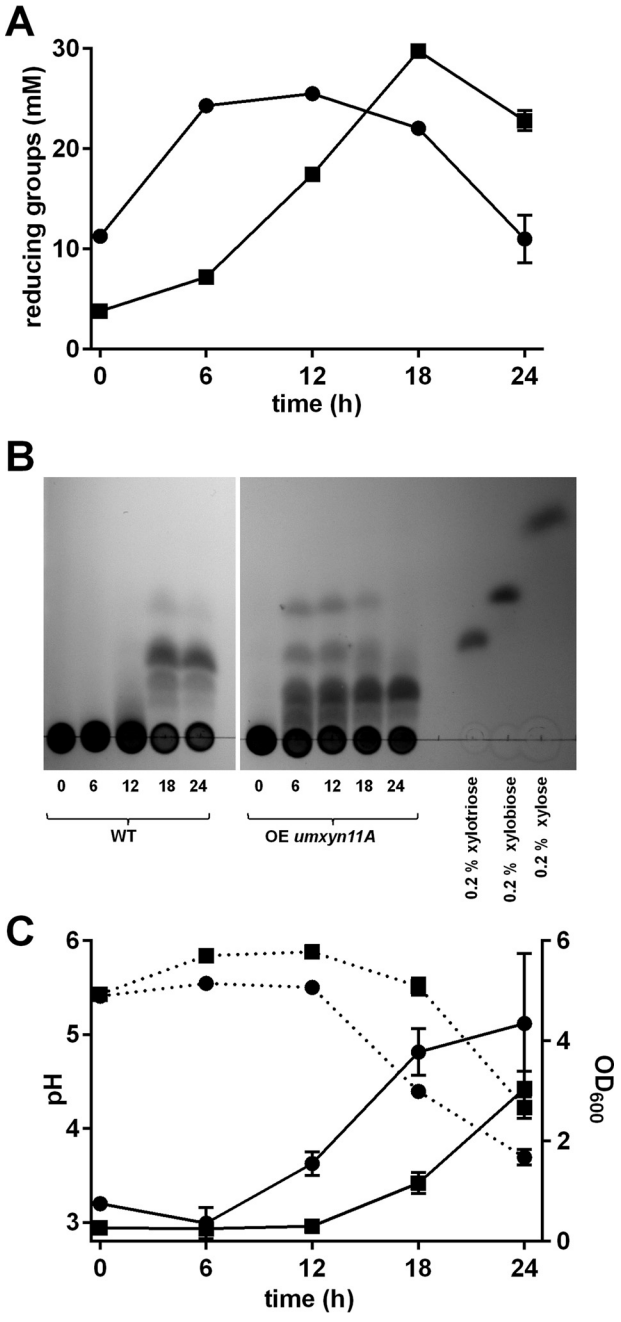

FIG 4 Cultivation of the MB215 wild type (■) and MB215P$_{oma}$xyn11A (●) in minimal birch-wood xylan medium. (A) Concentration of reducing groups was determined by a DNS assay. (B) Products of xylan hydrolysis in culture supernatants of the MB215 wild type (WT) and the MB215P$_{oma}$xyn11A overexpression strain (OE *umxyn11A* [i.e., *U. maydis xyn11A*]) detected in cultures grown on birch-wood xylan at different time points (in hours) by TLC. The figure is a composite of two parts of the same TLC, in which lanes not relevant to the study were removed for clarity. (C) pH profile (dashed lines) and growth curve (solid lines). Values are the arithmetic mean of four biological determinations. Error bars indicate deviations from the mean ($n = 4$).

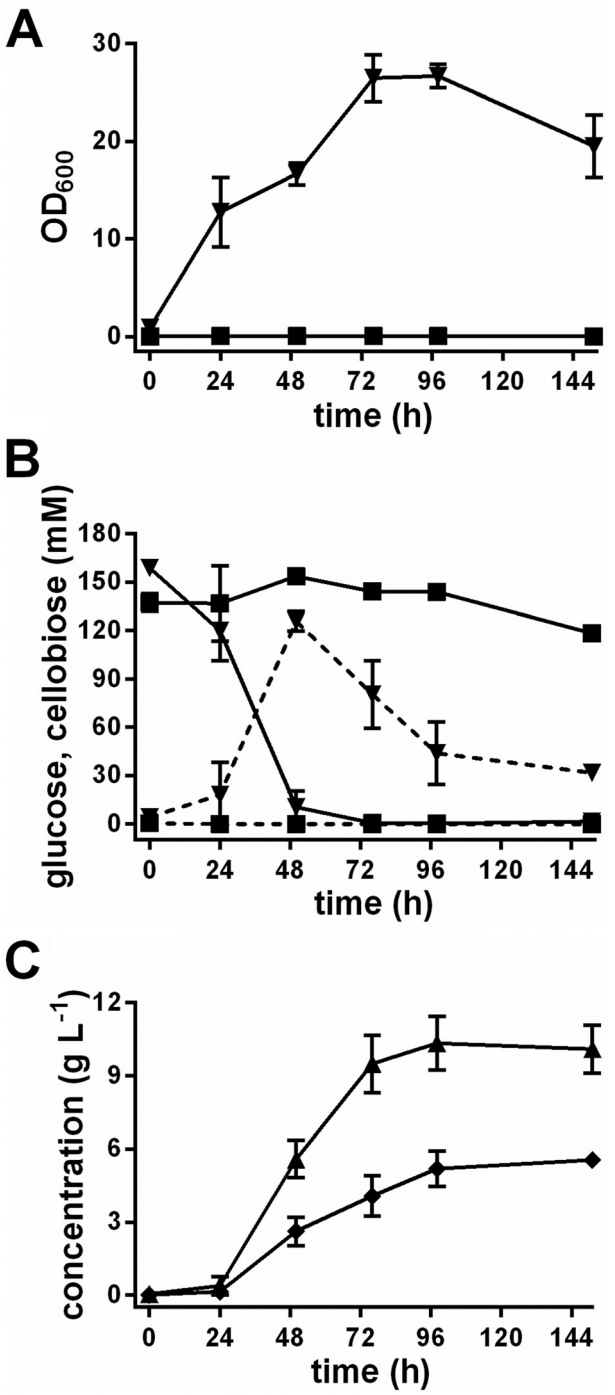

FIG 5 Cultivation of the MB215 wild type (■) and the MB215P$_{oma}$bgl1 overexpression mutant (▼) in minimal cellobiose medium. (A) Growth curve. (B) Concentration of glucose (dashed lines) and cellobiose (solid lines) during cultivation of the strains in minimal cellobiose medium. (C) Production of malate (▲) and itaconate (◆) from cellobiose by MB215P$_{oma}$bgl1. Values are the arithmetic mean of four biological determinations. Error bars indicate deviations from the mean ($n = 4$).

DISCUSSION

Relevant CAZymes can be activated in the yeast form. Here, we provide successful examples for the artificial activation of CAZymes in axenic culture. For all generated strains, transcription levels of the deregulated genes were strongly induced when the P$_{oma}$ promoter was used. In the corresponding strains, elevated enzyme activities were detected for three different enzymes.

It is of note that several overexpression strains did not have any

observable effect on the expected enzyme activities (see Table S2 in the supplemental material). Most of those constructs contained the P_{otef} promoter, while the P_{oma} promoter did lead to elevated enzyme activity. Quantitative real-time PCR suggests that although the activity of P_{otef} strains was higher than that in the wild type, it may still be too low to allow detection of the corresponding enzyme activity. Conversely, attempts at overexpressing other CAZyme genes did not lead to viable transformants. When P_{oma} was used, its activity might simply be too high, possibly resulting in an overload of the protein secretion system, followed by activation of the unfolded protein response (64, 65). Apparently, strict individual control of the expression levels of these secreted enzymes is necessary. Hence, future efforts will concentrate on the identification of novel promoters that harbor different intermediate activities during the yeast-like growth of *U. maydis*. Alternatively, inducible promoters could be utilized. Several of these exist in *U. maydis*, including a tetracycline-regulated system that is currently being developed to also allow induction upon the addition of tetracycline (T. Hyland, B. Steuten, J. Kämper, and M. Feldbrügge, unpublished data; see references 66 and 67). This would provide a means to fine-tune the expression of genes for hydrolytic enzymes in a way that allows for a good balance between high activity, efficient secretion, and cell growth. In this respect, the enhancement of the stability and amount of the secreted enzymes by reducing the proteolytic potential (47) and manipulating the unfolded protein response will also be a focal point (68, 69).

Strategies to expand substrate repertoire and complexity. In this proof-of-principle study, simple substrates and strains harboring single activated CAZymes were investigated. In the next step, the degradation of more relevant and complex lignocellulose-derived substrates, like (semi)crystalline cellulose, will be attempted. More complex substrates, however, will need the simultaneous action of different enzymes (70). Efficient degradation of cellulose, for example, requires the simultaneous action of endoglucanase, β-glucosidase, cellobiohydrolase I and II, and lytic polysaccharide monooxygenase, as well as several accessory factors (10, 71). Also, complete hemicellulose hydrolysis is based on the action of multiple enzymes (72). In this respect, it is noteworthy that we observed growth on xylan upon the sole overexpression of the endoxylanase gene *xyn11A*. Likely, the initial degradation of xylan into oligomers suffices to trigger native induction pathways for the complementary set of xylanolytic enzymes. However, TLC analysis showed that the xylan was not completely degraded (Fig. 4B), clearly indicating room for improvement.

On CMC and RAC, we observed a synergistic effect when mixing supernatants of strains with activated β-glucanase and endoglucanase. This indicates the potential for combining strains with different hydrolytic abilities. On CMC, for example, after 8 h, no further glucose production was observed in the assay with the combined secretomes of the Egl1 and Bgl1 strains (Fig. 3A), even though only a fraction of the added CMC was converted to glucose. This is likely caused by product inhibition of the activated CAZymes also apparent in the sharp decrease in activity over time. Consolidated bioprocessing can greatly reduce the negative effect of such product inhibition since the end products of (hemi)cellulose degradation are constantly being consumed by the biomass (73, 74). Furthermore, based on the predicted activities of the overexpressed enzymes, we would expect cellulose oligomers rather than glucose to accumulate in these experiments. Unfortunately, these substances could not be determined in the performed assays. However, generating strains expressing, e.g., cellobiohydrolases should again allow a more complete degradation and accumulation of higher glucose concentrations (10).

In the future, mixed cultures of two and more activated *U. maydis* strains will be adopted to enhance the degree of degradation of relevant (hemi)cellulose substrates. This strategy might also be a good means to provide flexibility by adapting the activated strains and their ratios depending on the substrate to be degraded. In *U. maydis*, it is furthermore possible to enable the simultaneous activation of multiple CAZymes in a single strain using resistance marker recycling (47, 75). That way, specialized strains for the degradation of different lignocellulose-building blocks could be developed.

Alternatively, it is conceivable to even further extend the concept of mixed cultivation and include other fungi or bacteria into the culture to exploit synergistic effects in biomass degradation, which are likely to occur when choosing organisms with complementary enzyme equipment. The potential of mixed cultures in biomass degradation has been described for diverse cases and mirrors nature where microbial communities cooperate in degrading plant matter (76, 77).

A potential alternative strategy is to artificially activate central transcriptional regulators that control multiple CAZy genes. Such regulators have been described, among others, for *Aspergillus niger* and *Neurospora crassa*, and could potentially activate a complete set of CAZymes simultaneously (10, 78, 79). Furthermore, potent heterologous enzymes, such as cellulases containing carbohydrate-binding domains (80), should be exploited to enhance the accessibility of the cellulose. These seem to be underrepresented in *U. maydis*. Importantly, *U. maydis* also possesses an unconventional secretion pathway which evades *N*-glycosylation, enabling the secretion of prokaryotic enzymes as well as fungal enzymes. Such a case is already known for the bacterial β-glucuronidase (GUS), and it was proven that unconventional secretion could be used to express the protein in its active form (26, 47, 81).

Besides looking only at the conventional CAZymes, additional focus should be paid on lignin-depolymerizing enzymes as well as potential accessory proteins, like expansins or swollenins (82). Recently, also enzymes lacking hydrolytic activity but revealing auxiliary activities that act in conjunction with CAZymes have been described (auxiliary activity [AA] class of redox enzymes [83]). These include lignin-degrading enzymes as well as lytic polysaccharide monooxygenases (LPMO) (84). In *U. maydis*, only one putative LPMO member of AA10, the copper-dependent LPMO, has been identified (85), suggesting that the insertion of additional foreign enzymes may be beneficial. Interestingly, putative oxidoreductases that are yet uncharacterized seem to be overrepresented in the *U. maydis* genome. A previous study hypothesized that the unexpectedly efficient biomass-degrading potential of the fungus might be attributed to these enzymes (43).

Potential of *U. maydis* for consolidated bioprocessing. *U. maydis* not only possesses promising enzymes for the degradation of lignocellulosic plant matter (43) but is also equipped for the production of valuable products, such as organic acids or biosurfactants (26). These features may allow its use for consolidated bioprocessing of lignocellulosic materials in the future. Importantly, engineering *U. maydis* for consolidated bioprocessing does not increase the risk of spreading the associated corn smut disease. Used strains are nonpathogenic as they are haploid and carry the

same mating type. Plant infection would require mating of haploid strains with compatible mating types. For production strains, it would be feasible to further decrease the risk by deleting the mating type genes at the *a* and *b* loci (30). This would result in very high safety conditions, since the resulting strains would be unable to proliferate by sexual reproduction even in the rare event of fermenter contamination with compatible wild-type strains or accidental strain release into the environment.

In this proof-of-principle study and without further optimization, we succeeded in producing about 5 g liter^{-1} itaconic acid and about 10 g liter^{-1} malate from cellobiose (Fig. 5). Current values, e.g., for itaconic acid achieved with industrial strains of *A. terreus*, are an order of magnitude higher. However, further development by the above-described strategies could still eventually yield a competitive system. In addition, the biosynthesis pathways for itaconic acid as well as for the potentially interesting glycolipids have been described in detail, and initial attempts of metabolic engineering have already demonstrated their potential to increase productivity (27, 86, 87).

In summary, the approach for converting simple plant cell wall components to organic acids described in this study utilizes all-native enzymes on both the substrate and the product side, ensuring maximal compatibility with the host and the possibility of generating "self-cloning" non-genetically modified organism (GMO) strains. Individual activation of hydrolytic enzymes allowed utilization and growth on novel substrates. If this concept can be expanded to more complex substrates, it may be conceivable in the future to design a consolidated bioprocess combining efficient biomass decomposition with product formation.

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L.M.B., M.F., N.W., and K.S. designed the study. E.G. and M.R. planned and conducted the wet-bench experiments. All authors contributed to the data analysis. N.W. and K.S. directed the project and prepared the manuscript with input from all coauthors.

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